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Mutagenicity of 1-Nitropyrene  
Metabolites from Lung S9

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## MUTAGENICITY OF 1-NITROPYRENE METABOLITES FROM LUNG S9

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### SUMMARY

The mutagenicity of 1-nitropyrene metabolites from rabbit lung S9 incubates was evaluated using the *Salmonella typhimurium* plate incorporation assay with strain TA98, with and without Aroclor-induced rat liver S9. The following metabolites were isolated, identified and quantitated by HPLC: 1-nitropyrene -4,5- or -9,10-dihydrodiol (K-DHD), *N*-acetyl-1-aminopyrene (NAAP), 1-aminopyrene (1-AMP), 10-hydroxy-1-nitropyrene, 4-, 5-, 6-, 8- or 9-monohydroxy-1-nitropyrene (phenols) and 3-hydroxy-1-nitropyrene. The predominant metabolites formed by lung S9 incubates were K-DHD, 3-OH-1-nitropyrene and phenols. All of the metabolites were mutagenic in the absence of the exogenous rat liver S9 metabolic activation system, and several, including two unidentified metabolites were more potent than the parent 1-nitropyrene. The mutagenicity of 3 of the metabolites (NAAP, 10-OH-1-nitropyrene and phenols) were enhanced by S9 while most of the other metabolites were less mutagenic in the presence of S9. These results indicate that lung tissue is capable of both oxidative and reductive metabolism which produced mutagenic metabolites, several of which were more potent than the parent compound, 1-NP.

### INTRODUCTION

Nitro-substituted polycyclic aromatic hydrocarbons (NO<sub>2</sub>PAHs) identified in xerographic toners [1,2], diesel particles [3,4] and ambient air (unpublished data) are potent bacterial mutagens [1]. Several NO<sub>2</sub>PAHs (e.g. 1-nitropyrene) have been shown to be mutagenic in mammalian cells [5,7] and carcinogenic in rodents [8]. Evidence is now accumulating that 1-nitropyrene is metabolized by mammalian systems in vivo [9] and in vitro [10-12,

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unpublished data] and that some of the metabolites produced are more potent mutagens than the parent compound 1-nitropyrene. In vitro studies performed in our laboratory using rabbit alveolar macrophages and respiratory tissues show that these cells and tissues metabolized 1-[ $^{14}\text{C}$ ]nitropyrene and that 1-[ $^{14}\text{C}$ ]nitropyrene or its metabolites bind to protein and DNA [11, unpublished data]. Lung and tracheal tissue produce metabolites that have undergone both nitro-reduction (e.g. producing NAAP) and ring oxidation (e.g. producing 1-nitropyrene phenols) (unpublished data). This paper reports data on the mutagenicity of 1-nitropyrene metabolites isolated from rabbit lung S9 incubates.

#### MATERIALS AND METHODS

##### *Animals*

New Zealand white male rabbits weighing 1.5–2 kg (Dutchland Laboratory animals, Denver, PA) were individually housed in a 12-h light/dark cycle animal room, maintained on antibiotic-free Purina Rabbit Chow (Ralston Purina, St. Louis, MO) and provided water ad libitum.

##### *Chemicals*

1-Nitro[4,5,9,10- $^{14}\text{C}$   $^{1/4}$ ]pyrene ( $^{14}\text{C}$ -1-NP), 59.5 mCi/mmol, prepared by nitration of [4- $^{14}\text{C}$ ]pyrene; unlabelled 1-NP; a mixture of 1-nitro (*cis*-4,5-dihydro-4,5-dihydroxy-) and (*cis*-9,10-dihydro-9,10-dihydroxy-) pyrene, K-DHD; and the isomers 3-, 4-, 5-, 6-, 8-, 9- and 10-hydroxy-1-NP were synthesized and characterized by Midwest Research Institute, Kansas City, MO, under EPA Contract No. 68-02-3177. NADP, glucose-6-phosphate dehydrogenase (Baker's yeast type VIII),  $\beta$ -glucuronidase (Sigma Type VII from *E. coli*) and 1-AMP (grade II) were purchased from Sigma Chemical Co., St. Louis, MO. NAAP was prepared by acetylation of 1-AMP (unpublished data). Unlabelled 1-NP was added to  $^{14}\text{C}$ -1-NP to adjust its specific activity to 10 mCi/ mmol. Ethyl acetate (EA), acetone (AC), methanol (MeOH) and methylene chloride ( $\text{CH}_2\text{Cl}_2$ ) were of spectrograde quality and purchased from Burdick and Jackson Laboratories, Muskegon, MI.

##### *Preparation of lung S9*

The lungs from 1 rabbit (94 g) were immediately removed following perfusion using a procedure previously described [11], placed in a beaker containing 500 ml of cold KCl (1.15%) and thoroughly minced. The KCl was decanted, the tissues washed twice with 500 ml of cold KCl (1.15%) and homogenized in 2 vols. of 0.25 M sucrose (185 ml) using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The homogenate was centrifuged at  $9000 \times g$  for 25 min at  $4^\circ\text{C}$ . The protein content of the resulting supernatant (S9) was determined by the method of Lowry et al. [13].

#### *Metabolism of $^{14}\text{C}$ -1-NP by lung S9*

$^{14}\text{C}$ -1-NP (2916 nmol; 81.0 nCi/ml) was added to 360 ml of lung S9 (5 mg/ml protein), diluted with Dulbecco's phosphate buffered saline (pH 7.4), and supplemented with 10 mM  $\text{MgCl}_2$ , 10 mM glucose 6-phosphate and 1.0 mM NADP. The above mixture was incubated in glass tubes (30 ml each) at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air for 45 min on a rocking platform. After min, 10 ml of ethyl acetate/acetone (EA/AC) (2:1) was added to each tube. The incubates were pooled, extracted 3 times with an equal volume of EA/AC and the pooled extracts evaporated to dryness at 37°C with a rotary evaporator. The residue was dissolved in 30 ml of 0.005 M phosphate buffer (pH 6.9) and incubated for 4 h at 37°C with  $\beta$ -glucuronidase (1000 units/ml). This mixture was extracted 3 times with EA/AC and the pooled extracts evaporated to dryness as described. The final residue was resuspended in 50% aqueous methanol for analysis by high pressure liquid chromatography (HPLC). The  $^{14}\text{C}$  content remaining in the aqueous phase and that associated with protein after repeated extraction with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (1:1) was quantitated by liquid-scintillation counting (Packard Instrument Co. model 460C, Downers Grove, IL).

#### *Analysis of 1-nitropyrene metabolites*

1-Nitropyrene metabolites were analyzed with a Varian Model 5060 high pressure liquid chromatograph equipped with a model CDS 401 Vista Data system (Varian Associates, Inc., Walnut Creek, CA). The columns used for analysis were a Zorbax ODS column (4.6  $\times$  250 mm, Dupont Co., Wilmington, DE) and a guard column (4.6  $\times$  50 mm) packed with pellicular ODS (Whatman, Inc., Clifton, NJ). The metabolites were eluted with a methanol/water gradient (60–100%, 1%/min) for 40 min at a flow rate of 0.8 ml/min. The eluate was monitored with a Varian Model 50 UV-Vis detector at 280 nm, and for fluorescence using a Model MPF 2A fluorescence spectrophotometer (Perkin Elmer Corp., Norwalk, CT; excitation, 360 nm; emission, 430 nm). The eluate was collected in 30-s fractions (Gilson B-200, Gilson Medical Electronic, Middleton, WI) and the  $^{14}\text{C}$  content determined by liquid scintillation counting. Figure 1 shows the elution pattern of the reference compounds co-eluted with the EA/AC soluble metabolites from lung S9. Metabolite fractions corresponding to peaks of radioactivity were pooled and re-chromatographed using the same gradient as described or with a different gradient (methanol/water, 75–100%, 1%/min) over 40 min at the same flow rate as described, to provide purer samples for bioassay.

#### *Mutagenicity*

Isolated metabolites were analyzed in duplicate at 5 dose levels (0.125–2.0 nmol/plate) for mutagenicity in *Salmonella typhimurium* strain TA98, in the presence and absence of hepatic S9 prepared from Aroclor-treated rats according to the method developed by Ames et al. [14], with minor modifications [15]. The strain TA98, which detects frame shift mutagens, was

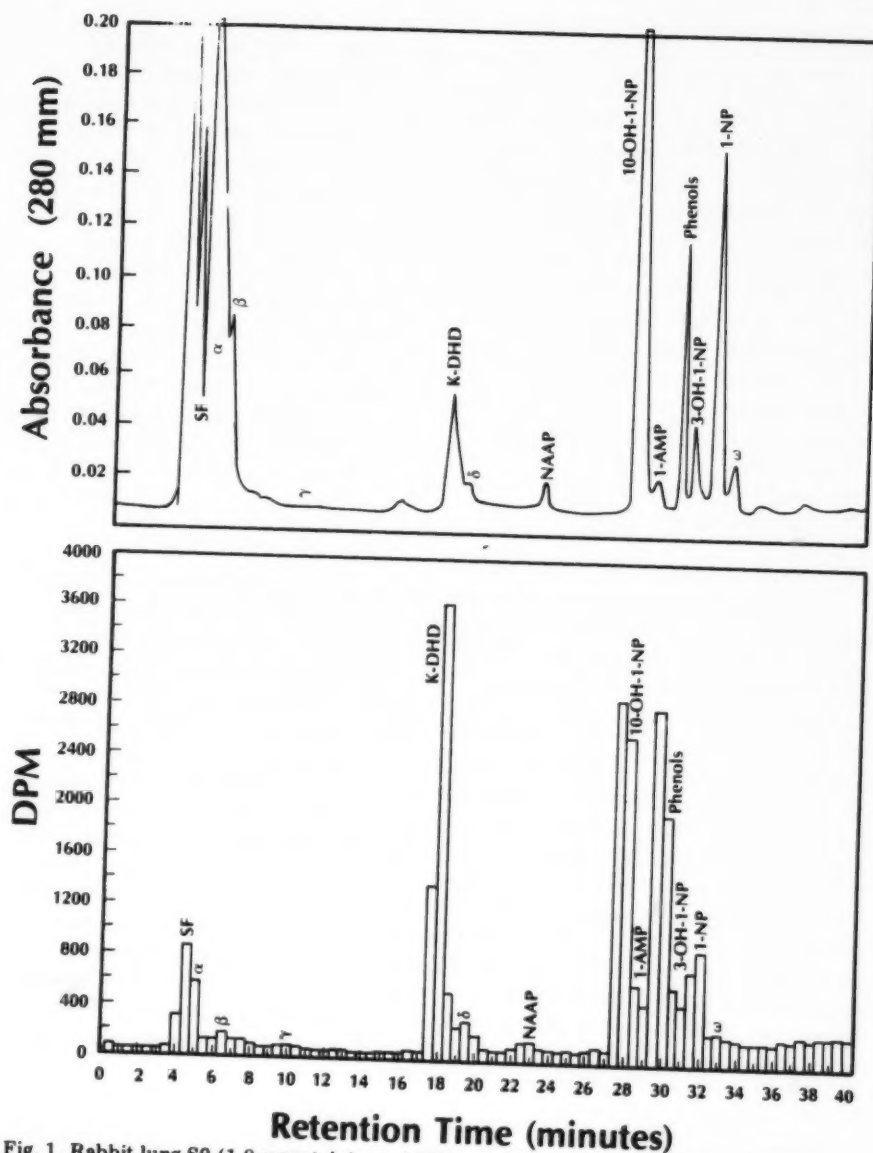


Fig. 1. Rabbit lung S9 (1.8 g protein) was incubated with 1-nitro[ $^{14}\text{C}$ ]pyrene ( $2773.0 \pm 223.2$  nmol) for 45 min. After extraction and concentration, a portion of the extract (10  $\mu\text{l}$ ) together with 10  $\mu\text{l}$  of unlabelled reference compounds were examined by HPLC, and the retention of the peaks of  $^{14}\text{C}$  radioactivity were compared to the absorbance of the reference compounds. (a) Absorbance of synthetic standards and metabolites at 280 nm. (b) Radioactivity.

selected because it is the most responsive of the 5 widely used Ames strains to 1-NP [1], and the quantities of metabolites which could be obtained were sufficient for evaluation in only 1 tester strain. 2-Nitrofluorene (without S9), 3.0  $\mu\text{g}/\text{plate}$  and 2-anthramine (0.5  $\mu\text{g}/\text{plate}$ ) with S9 were used as positive controls. Historical values in our laboratory are 180–400 revertants/plate and 700–1000 revertants/plate, respectively and experiments falling outside these ranges were rejected. Mutagenicity was expressed as revertants per nanomole of compound based on  $^{14}\text{C}$  content, using linear regression analysis derived from the initial linear portion of the dose response curve.

## RESULTS

The metabolites formed by the lung S9 incubates (Fig. 1, Table 1) were identified by their co-elution with authentic standards, retention time and

TABLE 1  
METABOLISM OF 1-NITRO[ $^{14}\text{C}$ ]PYRENE AND MUTAGENICITY OF THE ISOLATED METABOLITES<sup>a</sup>

| Metabolite fraction<br>(By HPLC) | Nanomoles<br>(mean $\pm$ S.E.M.) <sup>b</sup> | Specific mutagenicity<br>(revertants/nmol) <sup>c</sup> |                 |
|----------------------------------|-----------------------------------------------|---------------------------------------------------------|-----------------|
|                                  |                                               | –S9                                                     | +S9             |
| Solvent front                    | 44 $\pm$ 1                                    | 55 $\pm$ 10                                             | 14 $\pm$ 6      |
| $\alpha$                         | 52 $\pm$ 4                                    | 554 $\pm$ 33                                            | 70 $\pm$ 1      |
| $\beta$                          | 16 $\pm$ 1                                    | 1248 $\pm$ 97                                           | 113 $\pm$ 4     |
| $\gamma$                         | 15 $\pm$ 0                                    | 168 <sup>d</sup>                                        | 44 <sup>d</sup> |
| K-DHD                            | 318 $\pm$ 25                                  | 373 $\pm$ 100                                           | 94 $\pm$ 24     |
| $\delta$                         | 19 $\pm$ 1                                    | 201 $\pm$ 50                                            | 94 $\pm$ 41     |
| NAAP                             | 22 $\pm$ 3                                    | 62 $\pm$ 6                                              | 347 $\pm$ 150   |
| 10-OH-1-NP                       | 19 $\pm$ 3                                    | 77 $\pm$ 7                                              | 215 $\pm$ 110   |
| 1-AMP                            | 67 $\pm$ 10                                   | 157 $\pm$ 20                                            | 151 $\pm$ 80    |
| Phenols                          | 364 $\pm$ 20                                  | 84 $\pm$ 6                                              | 168 $\pm$ 57    |
| 3-OH-1-NP                        | 279 $\pm$ 10                                  | 584 $\pm$ 27                                            | 222 $\pm$ 24    |
| 1-NP                             | 413 $\pm$ 45                                  | 315 $\pm$ 35                                            | 227 $\pm$ 30    |
| $\omega$                         | 20 $\pm$ 4                                    | 126 $\pm$ 16                                            | 142 $\pm$ 17    |
| Total metabolites <sup>e</sup>   | 2360                                          |                                                         |                 |

<sup>a</sup> $^{14}\text{C}$ -1-NP (2773.0  $\pm$  223.2 nmol) was incubated with rabbit lung S9 preparation (1.8 g protein) for 45 min as described in Materials and Methods.

<sup>b</sup>Results are expressed as mean nmol formed by 1.8 g protein in 45 min from duplicate experiments.

<sup>c</sup>Revertants/nmol  $^{14}\text{C}$  derived material determined by the reverse mutation plate incorporation assay in TA98 with (+S9) and without (–S9) Aroclor induced rat liver S9 as described in Materials and Methods. Data is the mean slope  $\pm$  S.E.M. from 2 separate assays.

<sup>d</sup>Single determination due to sample size.

<sup>e</sup>Total metabolites includes those bound to protein (669  $\pm$  80 nmol) and those remaining in the aqueous phase (437  $\pm$  280 nmol) after repeated extractions.

comparison with 1-NP metabolites described previously [9,12, unpublished data]. The predominant metabolites formed were the 1-nitropyrene -4,5- or -9,10-dihydrodiol (K-DHD), 4-,5-,6-,8- or 9-monohydroxy-1-nitropyrene (phenols) and 3-hydroxy-1-nitropyrene (3-OH-1-NP). Hydrolysis of an aliquot of the K-DHD metabolite in HCl (1 M) produced derivatives that were chromatographically indistinguishable from the metabolites identified as phenols and trace amounts of 3-OH-1-NP (data not shown). This evidence supports the identification of this metabolite of 1-NP as K-DHD. The overall total metabolism by lung S9 was greater than the proportion of 1-NP consumed by previous lung tissue incubates (28%) (unpublished data). A significant portion of the total  $^{14}\text{C}$  ( $2773 \pm 223$  nmol) was irreversibly bound to the lung S9 protein (24%) or remained in the aqueous phase (16%), suggesting the capacity of lung S9 to produce metabolites that bind to cellular macromolecules.

All of the metabolites as indicated in Table 1 were mutagenic in the absence of the exogenous rat liver S9 metabolic activation system. Three of the metabolites ( $\alpha$ , 3-OH-1-NP and  $\beta$ ) exhibited mutagenicity that was 2–4 times more potent than the unmetabolized 1-NP based on slope values derived from the linear portion of the dose response curve. The mutagenicity of three of the metabolites (NAAP, 10-hydroxy-1-nitropyrene (10-OH-1-NP) and phenols) as enhanced by S9, while most of the other metabolites were less mutagenic in the presence of S9. Table 1 does not include the non-extractable metabolites bound to protein and in the aqueous phase. These metabolites which represent about 47% of the detectable products were not evaluated for mutagenicity at this time.

#### DISCUSSION

This study demonstrates that 1-NP is metabolized by rabbit lung S9 to produce both oxidized and reduced metabolites, several of which are more mutagenic than the parent compound. It is significant, that the lung S9 produced higher proportions of K-region dihydrodiols (13.5% of the total metabolites) from 1-NP than observed for Aroclor induced liver S9 (unpublished data). El-Bayoumy and Hecht [12] also reported the identification of a K-DHD liver metabolite of 1-NP (4,5-dihydro-4,5-dihydroxy-1-nitropyrene) but the quantities produced were not sufficient to assay for mutagenicity. The K-DHD we have isolated from lung S9 was mutagenic in the absence of S9 and similar in mutagenicity to the parent 1-NP. Rat lung microsomal incubations of benzo[a]pyrene also produced higher quantities of K-region dihydrodiols per milligram protein than did corresponding rat liver microsomal preparations [16]. The formation of oxidative metabolites from  $\text{NO}_2$ PAHs has also been observed when 1-NP [12] and 6-nitrobenzo[a]pyrene [17] were metabolized by liver microsomal preparations and liver S9.

Several of the phenolic lung metabolites, 3-OH-1-NP and phenols (containing 6- and 8-OH-1-NP), reported here were similar in quantity and muta-

genicity to those previously reported [12, unpublished data] as liver metabolites. We also have identified and isolated for the first time an additional phenol, 10-OH-1-NP, which showed enhanced mutagenicity in the presence of S9.

The rabbit lung S9 incubations with 1-NP also produced metabolites in which the nitro group had been reduced. These included NAAP and 1-AMP which were present at approximately one-tenth the concentration of the oxidized metabolites. Aroclor induced rat liver preparations also produced these reduced metabolites but in relatively higher proportions to the oxidized metabolites (unpublished data). The reduced metabolites, NAAP and 1-AMP, isolated from rabbit lung had specific mutagenicities comparable to the synthetic standards (unpublished data). The NAAP, an acetylation product of 1-AMP, when purified, is mutagenic in *S. typhimurium* TA98 only in the presence of S9 (unpublished data). The NAAP fraction isolated from rabbit lung appeared to contain some direct-acting mutagenic components, however, the mutagenicity was greatly enhanced (over 5-fold) by the addition of S9.

In addition to the previously identified metabolites, several more polar ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,) and one less polar ( $\omega$ ) unidentified metabolites were found. The two unidentified metabolites designated as  $\alpha$  and  $\beta$  were more mutagenic than 1-NP in the absence of S9. Based on their retention time and direct-acting mutagenicity it is speculated that these are highly polar oxidative metabolites with an intact nitro substituent. The  $\omega$  metabolite appears to be similar in retention time and mutagenicity to 1-nitrosopyrene, a postulated intermediate in the reduction of 1-NP [12,18]. The isolation, characterization and identification of these metabolites is in progress.

This evidence for the metabolism of 1-NP by lung S9 and the mutagenicity of these metabolites together with the previously reported DNA binding of 1-NP in lung tissue [11] provides strong evidence for the genotoxicity of 1-NP in the mammalian lung.

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